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A Diagnostic EIA for Detection of the Prevalent SRSV Strain in United Kingdom Outbreaks of Gastroenteritis

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Small round structured viruses (SRSVs) are the major cause of outbreaks of gastroenteritis in the UK. Diagnosis is problematic due to insensitive electron microscopy (EM) or technically demanding reverse transcription polymerase chain reaction (RT-PCR) techniques. We have studied outbreaks of non-bacterial gastroenteritis using an EIA based upon recombinant capsid protein from the currently prevalent circulating strain of SRSV (Lordsdale Genotype II) and compared its performance against EM and RT-PCR assays. Faecal specimens sent to the Bristol Public Health Laboratory for outbreak investigation from December 1996 to December 1997 were applied retrospectively to the SRSV EIA and results compared with the routine EM and RT-PCR that had been carried out prospectively. Overall, the three tests identified SRSVs in specimens from 70% of the outbreaks (213/305) investigated. Of the 213 total positive outbreaks, the EIA identified 71%, that compared favourably with EM (63%) and RT-PCR (84%). The Lordsdale Genotype II SRSV EIA provides a simple cost-effective assay that will for the first time make detection of currently circulating SRSV strains associated with UK outbreaks available to all routine laboratories. The EIA format makes the assay widely applicable to non-specialist laboratories, unlike the RT-PCR assay, and the improved sensitivity over EM will allow successful screening of UK outbreaks alongside commercial EIAs currently available for adenovirus, astrovirus and rotavirus. Furthermore, the assay will allow rapid identification of emerging SRSV strains. ***J. Med. Virol. 61:132–137, 2000.*** © 2000 Wiley-Liss, Inc.

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INTRODUCTION

The small round structured viruses (SRSVs) are the major cause of outbreaks of non-bacterial gastroenteritis. The viruses are often termed Norwalk or Norwalk-like viruses following their original identification in an outbreak in Norwalk, Ohio [Kapikian et al., 1972]. Since then, outbreaks have been described worldwide with the causative strain assuming the name of the outbreak location [Caul, 1996a,b] and SRSVs have now been classified within the *Caliciviridae* family.

SRSVs usually cause relatively mild gastroenteritis but the combination of a low infectious dose and symptoms that include projectile vomiting leads to large outbreaks arising from secondary spread. In semi-closed communities, most notably hospitals and residential homes for the elderly, outbreaks can involve both patients and staff, with consequent management problems caused by ward closures [Adak et al., 1991; Jiang et al., 1996; Dedman et al., 1998]. Community outbreaks caused by contaminated water [Goodman et al., 1982; Gray et al., 1997] or food [Riordan et al., 1984; Andersen et al., 1996] have also been reported and the financial consequences can be measured in lost working days. In the leisure industry, cruise ships and holiday centres provide further examples of "captive audiences" in that viral spread is facilitated [McAnulty et al., 1993; Koo et al., 1996; McEvoy et al., 1996].

In the UK, diagnosis is made by electron microscopy (EM), because SRSVs cannot be cultured in vitro [Dedman et al., 1998], but the low level of viral shedding and the relative insensitivity of EM hampers outbreak diagnosis. More recently, the complete genome characterisation of SRSVs [Jiang et al., 1993; Lambden et al., 1993; Dingle et al., 1995] opened the way for more sensitive RT-PCR methodologies [Green et al., 1995].

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SRSVs can be divided into two genetic groups, type I [Lambden et al., 1993] and Type II [Dingle et al., 1995]. The SRSV genome sequences are highly variable, and consequently the genetic groups are broad [Dingle et al., 1995; Green et al., 1997b]. Routine surveillance by RT-PCR cannot be carried out in most diagnostic laboratories and is therefore carried out in specialist centres. The need for rigorous extraction procedures to purify viral RNA also limits the numbers of clinical specimens that can be processed at a time. Consequently, the large numbers of specimens entering the laboratory in the winter months could easily overwhelm diagnosis by molecular methods.

Expression of the Norwalk virus capsid gene in insect cell culture produced virus like particles (VLPs) [Jiang et al., 1992] with antigenic properties compatible with those of the native virus. Genogroup II SRSV capsids have also been expressed as VLPs including Mexico [Jiang et al., 1995b] Toronto [Leite et al., 1996] and Hawaii viruses [Green et al., 1997a]. Antisera raised to VLPs have been used to develop antigen capture EIA assays and then applied to the detection of SRSVs in faecal specimens [Jiang et al., 1992, 1995a]. The EIA assays are strain specific and because all the above viruses are not predominant in the UK [Green et al., 1994] have proved to be of little value in routine diagnosis [Jiang et al., 1995c; Cubitt and Jiang, 1996]. In this study the development is described of an EIA assay based upon Lordsdale virus [Dingle et al., 1995], the predominantly circulating SRSV strain in the UK and applied it retrospectively to outbreak specimens received in the laboratory over a 1 year period in 1996–97.

METHODS AND MATERIALS

Viruses and Clinical Specimens

Lordsdale virus was identified and characterised after a large hospital outbreak of SRSV in March 1993 [Dingle et al., 1995]. Clinical specimens used in our study were from outbreaks of gastroenteritis primarily in the SW of England between December 1996 and December 1997. Faecal specimens were emulsified as approximately 10–20% suspensions in Eagle's minimal essential medium and the same sample used for solid phase immune electron microscopy (SPIEM), reverse transcription polymerase chain reaction (RT-PCR) and SRSV Enzyme-linked Immunosorbent Assay (EIA).

Cloning Sequencing Expression and Purification of Lordsdale VLPs

Lordsdale virus capsid gene cDNA was cloned into a baculovirus expression vector as described previously [Dingle et al., 1995]. VLPs were expressed in Trichoplusia ni (High Five cells) and purified on CsCl gradients [Pelosi et al., 1999].

Immunization Protocol

Antisera were raised in rabbits and laying hens by injection with purified Lordsdale VLPs in adjuvant essentially as described previously [James et al., 1998].

Solid Phase Immune Electron Microscopy (SPIEM)

SRSVs were captured onto formvar/carbon grids coated with anti-human IgG and human convalescent serum as described previously [Green et al., 1994] and then viewed using a Phillips 420 electron microscope (×62,500).

RT-PCR and DNA Sequencing

Diagnostic RT-PCR was carried out using RNA polymerase gene primers [Green et al., 1995] as described previously [Brugha et al., 1999]. DNA sequence was obtained by firstly optimising magnesium ion concentration for PCR using capsid gene primers before direct sequencing of the 223bp capsid gene amplicon using the Applied Biosystems 373A automated sequencer as described previously [Green et al., 1997b].

Immunoblotting

Faecal emulsions were analysed by SDS PAGE, blotted and tested for the presence of SRSV capsid protein using rabbit immune antisera as described previously [James et al., 1998].

Enzyme-Linked Immunosorbent Assay

Polyvinyl plates were coated by incubating 50 μl rabbit pre- and post-immune antisera (1:4,000) in coupling buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) overnight at 4°C. Excess coating antibodies were aspirated and the wells blocked by incubating with 300 μl of PBS/5% skim milk for 1 hour at room temperature. The wells were washed 4 times with PBS Tween20 (0.05% v/v) and finally 90 μl of PBS/1% skim milk was loaded into each well. Ten μl of faecal emulsion was added to pairs of wells (pre- and post-immune) and incubated at room temp for 90 minutes. Subsequently, the plate was washed 4 times with PBS Tween before 50 μl of chicken anti-SRSV IgY (1:5,000 in PBS/1% skim milk) was loaded into each well. The plates were incubated at room temperature for 1 hour, washed 4 times with PBS Tween and 50 μl goat anti-chicken antibody conjugated to horse radish peroxidase (1:3,000 in PBS-1% skim milk, Dynex Technologies) was applied to each well, followed by incubation for 1 hour at room temperature. Finally, the plate was washed four times in PBS Tween before development with 50 μl substrate (0.1% sodium acetate pH6.0, 1% tetra methyl benzidine, 0.01% H_2O_2) at room temperature for 10 minutes. The reaction was stopped by addition of 50 μl of 1.5 M H_2SO_4 and OD values obtained at 450 nm wavelength. Specimens with post-immune A_{450} values greater than twice the pre-immune and also greater by more than 0.1 OD unit were scored as positive.

RESULTS

Specimens Tested

Faecal specimens (2,030) were received in the laboratory from December 1996 to December 1997 derived from 305 outbreaks of gastroenteritis in SW England.

TABLE I. Summary of SRSV Detection in Outbreaks by EIA, SPIEM and RT-PCR

			Outbreaks	
EIA	EM	PCR	Number	%
+	–	–	12	3.9%
–	+	–	14	4.6%
–	–	+	25	8.2%
+	+	–	8	2.6%
+	–	+	41	13.4%
–	+	+	23	7.5%
+	+	+	90	29.5%
–	–	–	92	30.2%

Specimens were screened by SPIEM and a representative number of samples from each outbreak (EM positive) were surveyed by RT-PCR to provide genotype information. Representative specimens from EM negative outbreaks were also screened by RT-PCR. All specimens were stored at 4°C and subjected to the SRSV EIA retrospectively. The average number of specimens per outbreak was 7 with a maximum of 84. A known panel of faecal emulsions containing other enteric viruses were examined by EIA for cross-reactivity.

Comparison of Assays

Ninety-two outbreaks (30.2%) were negative by all tests, whereas the remainder were positive by one or more of the tests in combination. 14 (4.6%) outbreaks were positive by EM only, 25 (8.2%) outbreaks by RT-PCR only and 12 (3.9%) outbreaks by EIA only. One hundred sixty-two outbreaks were positive by two or more of the assays. In pairwise combinations, RT-PCR failed to identify 8 outbreaks detected by the two other assays, and similarly 23 were not detected by EIA and 41 outbreaks not detected by EM. Ninety (42.3%) of the 213 positive SRSV outbreaks were identified by all three tests in agreement (Table I). Within some outbreaks, the three tests identified SRSVs in different specimens. This probably reflects differing properties for individual specimens for each test e.g., RT-PCR is prone to inhibitors, EM is relatively insensitive whereas the EIA requires structurally competent and reactive viral capsid protein. In general, EIA and RT-PCR were more sensitive at identifying SRSV outbreaks, with more specimens per outbreak detected by these tests. In the case of RT-PCR the apparent increase in sensitivity may be biased because in some cases EM positive samples were specifically targeted to provide genotype information. Consequently, a higher positivity rate may merely reflect an increased positivity in the number of samples being tested by RT-PCR.

SRSV Genotyping

The RT-PCR assay using the polymerase gene primers was able to ascribe genotype information to most of the SRSV outbreaks. Of the 179 PCR positive outbreaks, 19 (10.6%) were identified as Genogroup I, 144 (80.4%) as Genogroup II and 15 (8.4%) were SRSV positive but the genogroup was unresolved by RT-PCR (Table II). In one outbreak, both Genogroup I and II viruses were identified in different patients. In this case the outbreak was associated with the consumption of shellfish in a restaurant. Other outbreaks have been documented previously that involve more than one SRSV strain [Sugieda et al., 1996; Brugha et al., 1999]. Mixed strain outbreaks are commonly associated with sewage contaminated bathing water [Gray et al., 1997] or undercooked shellfish that filter feed and concentrate the virus from contaminated water sources [Wanke and Guerrant, 1987; Dowell et al., 1995; Sugieda et al., 1996; McDonnell et al., 1997; Slomka and Appleton, 1998]. Consequently, the circumstances of the mixed strain outbreak in this study was consistent with those reported previously.

TABLE II. Genogrouping of the SRSV Outbreaks by RT-PCR Using RNA Polymerase Primers

	Number	%
Genogroup I	19	6.3%
Genogroup II	144	47.4%
Unresolved	15	4.9%
Mixed	1	0.3%
Neg	126	41.1%

Performance of the EIA

The specificity of the SRSV EIA was confirmed using a panel of clinical specimens containing other gastroenteritis viruses (astrovirus, adenovirus and rotavirus). No cross-reaction with any of these other viruses was observed. Overall the three tests identified SRSVs in 70% of the possible SRSV outbreaks. Individually, EIA was able to give a positive result in 71%, SPIEM in 63% and PCR in 84% of the positive outbreaks. Assuming the other outbreaks were negative for SRSV (negative in all three tests), the EIA was able to detect 71% of the SRSVs circulating in the South West of England during the study. Despite the high specificity of the Genogroup II Lordsdale virus EIA, five outbreaks were identified by the EIA although they were designated genogroup I by RT-PCR. It is possible these particular strains possess a common epitope identified by the Lordsdale antisera although more detailed analyses will be needed to characterise the nature of the EIA reactions with these viruses.

Characterisation of EIA Negative/PCR Positive Outbreaks

All EIA assays based upon recombinant SRSV VLPs described previously have been shown to be strain specific [Jiang et al., 1995c; Cubitt and Jiang, 1996; Hale et al., 1999]. Our EIA using Lordsdale recombinant capsids also shows this high specificity. In tests with a range of partially sequenced SRSV strains genetically closely related but distinct from Lordsdale virus, the EIA failed to detect these strains (data not shown). To broaden the reactivity ('catch-all') of our SRSV EIA, it is essential for continued molecular epidemiological surveillance to identify emerging variants that may become predominant strains in the UK. To this end, we

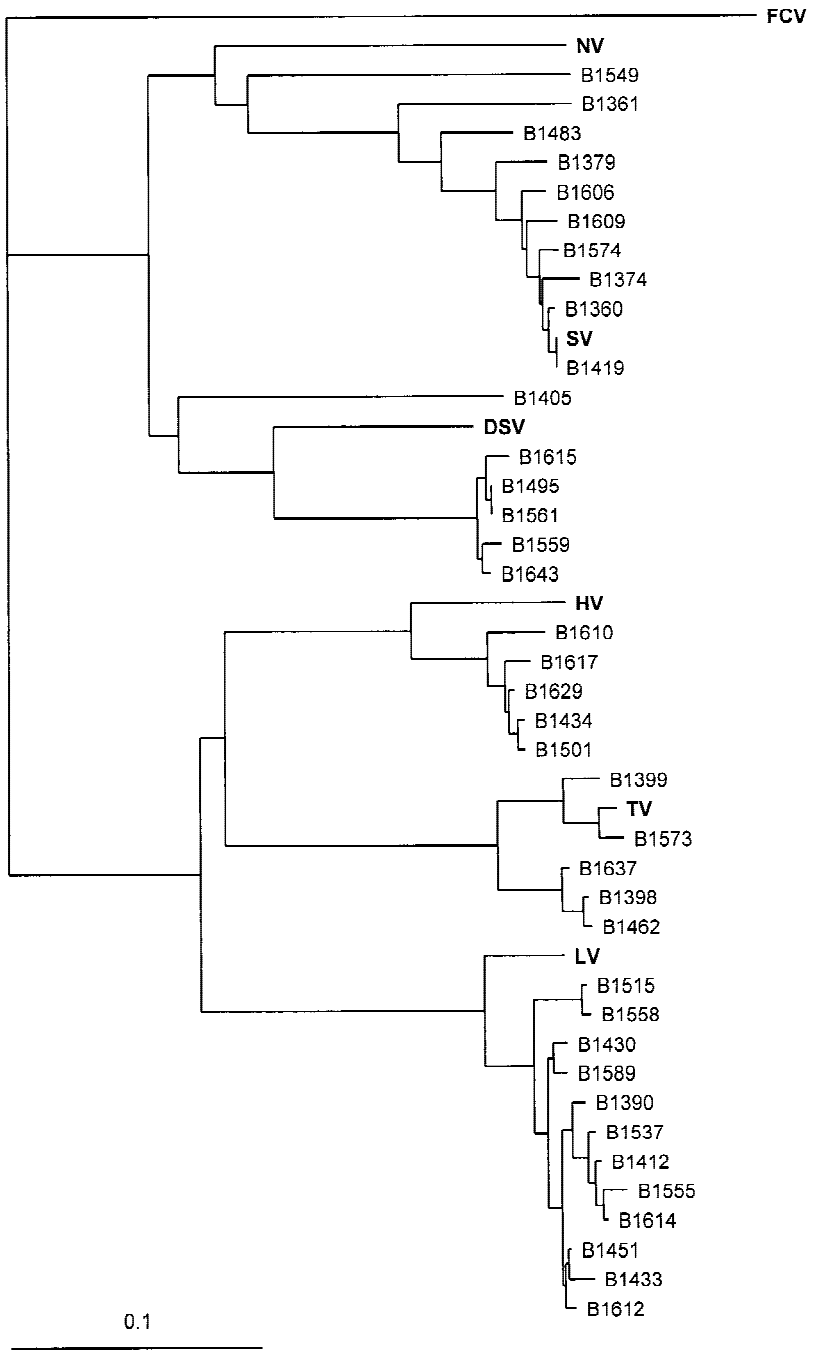

Fig. 1. Dendrogram showing the phylogenetic relationship of SRSV capsid sequences obtained from outbreaks not detected by EIA. Typical members of Group I (NV, Norwalk virus [M87661]; SV, Southampton virus [L07418] and DSV, Desert Shield Virus [U04469]) and II (HV, Hawaii virus [U07611]; TV, Toronto virus [U02030] and LV, Lordsdale virus [X86557]) and FCV, feline calicivirus (M86379) for comparison are indicated in bold type. The scale of the abscissa to the connecting node is proportional to genetic distance between sequences. The scale represents nucleotide substitutions per site.

have characterised strains that were circulating during this study, that evaded capture by our EIA. A region of the capsid gene was amplified by PCR and sequenced from those outbreaks that were RT-PCR positive, but negative by EIA. In total 38 such outbreaks were analysed, and comprised 16 Genogroup I viruses and 22 Group II viruses (Fig. 1). Ten of the Group I viruses clustered with Southampton virus (76–98.8% identity) whereas the other six clustered with Desert Shield virus (74.5–83.4% identity). Among the 22 Genogroup II viruses, 12 clustered with Lordsdale virus (90.3–94.3% identity), 5 with Toronto virus (90.3–97.8% identity) and 5 with Hawaii virus (87.2–90.8%). Although the sequence of the capsid amplicons from some of these strains were most similar to Lordsdale virus, they were not identical (<95% nucleotide identity). It is quite possible that amino acid differences in these capsids are sufficient to alter the antigenic structure such that the Lordsdale specific antisera cannot bind these epitopes.

Confirmation of SRSV EIA Positives (PCR and EM Negative Outbreaks)

In 35 specimens from 12 outbreaks, the EIA yielded a positive result in the absence of corroborative evi-

dence from any of the other tests. To verify that EIA positive samples from these outbreaks were not false positives, a representative specimen from each of these outbreaks was analysed by immuno-blotting to determine the identity of the reactive protein. The presence of a 60 kDa protein in specimens from 4 of these outbreaks confirmed the presence of the SRSV capsid protein. The inability to detect the 60 kDa species in samples from the other 8 outbreaks is not unusual and probably reflects the lower sensitivity of the blotting technique. Previously we have found EIA positive samples (confirmed by EM or PCR) that are negative by Western blotting (data not shown). Not all the specimens from these 12 EIA positive outbreaks had been examined by RT-PCR because of the logistics of processing large numbers of samples. In the light of our EIA results, further samples from these outbreaks were examined by RT-PCR, targeting the EIA positive specimens (also including the immunoblotted specimens). SRSV amplicons were detected in samples from four of the outbreaks (one of that was positive in the immuno-blot). Thus, in total 7 out of the twelve EIA positive outbreaks could be confirmed by other methods. The 5 outbreaks that remained unconfirmed in the laboratory were negative bacteriologically and were clinically and epidemiologically consistent with SRSV gastroenteritis. Failure to detect the virus by the other methodologies in samples from these 5 outbreaks may be due to a number of factors. Primer-template mismatches, the presence of inhibitors (RT-PCR), low levels of virus or timing of clinical samples (EM) are possible causes of false negatives. Alternatively, although the RT-PCR and EM assays require whole virus particles either to protect labile RNA genome or to provide morphologically identifiable particles respectively, the EIA additionally detects soluble capsid protein . Thus, it is possible that in some specimens the soluble capsid protein will be present in larger quantities [Jiang et al., 1992] than whole virus giving the EIA assay a diagnostic advantage. Only 2.3% of the SRSV outbreaks diagnosed by our EIA were unconfirmed by other tests.

DISCUSSION

The diagnosis of outbreaks of SRSV has been hampered for a long time by the inability to culture these viruses in vitro. The assays available to diagnose SRSV outbreaks are limited in sensitivity (EM) or are complex (RT-PCR) and cannot be carried out in most routine laboratories. SRSV EIAs described previously have been shown to be highly strain specific and therefore of limited diagnostic value because the strains described in their reports are not prevalent currently in the UK [Jiang et al., 1995c; Cubitt and Jiang, 1996]. Therefore, we chose to develop an SRSV EIA based upon Lordsdale virus capsid protein because our molecular surveillance has shown that the Lordsdale virus cluster is most prevalent in the UK [Green et al., 1994, 1997b]. Since then, other groups have confirmed the relatively high prevalence of this strain in the UK [Maguire et al., 1999]. More recently, the worldwide distribution of the Lordsdale virus has been documented [Vinje and Koopmans, 1996; Fankhauser et al., 1998; Wright et al., 1998; Noel et al., 1999]. The EIA based on Lordsdale (Genotype II) virus capsid has for the first time produced an assay that is relevant not only to the currently predominant SRSV involved in UK outbreaks, but also worldwide. By comparison with EM and RT-PCR assays, the EIA is cost-effective, sensitive and allows larger numbers of specimens to be screened, that is particularly important in the winter months at the height of the SRSV season. The SRSV EIA complements the commercial EIAs available for astrovirus, adenovirus and rotavirus. The ability to identify outbreaks locally allows a rapid diagnostic response allowing appropriate management of outbreaks in hospitals and other semi-closed communities. Although we recognise the high specificity of our EIA and that changes in prevalence of circulating strains will possibly alter its diagnostic value, the EIA does provide currently an effective pre-screen of non-bacterial gastroenteritis outbreaks. Negative outbreaks would be forwarded to specialised centres for further screening by RT-PCR and electron microscopy. In this way the EIA will permit the monitoring of new trends in circulating virus strains and provide an early warning for the need to further develop the assay. In the meantime the EIA will provide important epidemiological data about that SRSV strains are circulating and allow identification of major epitope changes in SRSVs. This feature is a critical part of the process of monitoring SRSV outbreaks. Continual molecular surveillance to characterise emerging strains will allow a structured approach to developing relevant antibodies for the future development of a “catch-all” EIA.

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